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APPLICATION FOR PATENT

TITLE: METHODS AND COMPOSITIONS FOR ENHANCED PLANT CELL TRANSFORMATION

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METHODS AND COMPOSITIONS FOR ENHANCED PLANT CELL TRANSFORMATION

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[0001] This application is a continuation-in-part of co-pending U.S. Serial No. 10/098,161 filed March 14, 2002, which claims priority from PCT/US00/25260, filed September 14, 2000 and U.S. Serial No. 60/154,158 filed September 15, 1999 now abandoned. The United States Government may have some rights to the invention disclosed due to partial support from the National Science Foundation (NSF) Grant Nos: IBN-9630779 and DBI-0110023.

BACKGROUND OF THE INVENTION

[0002] The invention relates enhanced *Agrobacterium*-mediated transformation frequencies of plants due to addition of histones to the plant to be transformed. Methods specific for enhancing monocot transformation frequencies are also disclosed wherein both histones and L-cysteine are factors.

[0003] *Agrobacterium tumefaciens* is a gram negative soil bacterium that has been exploited by plant biologists to introduce foreign DNA into plants. The routine, efficient *Agrobacterium*-mediated transformation of dicotyledonous plants was first reported in the mid 1980's. Because monocotyledonous plants are not natural hosts for *Agrobacterium tumefaciens*, the development of transformation systems using this vector for monocots lagged that of dicots. Direct DNA delivery techniques including electroporation, microprojectile bombardment, and silicon carbide fiber treatment were developed for monocot transformation as alternatives to *Agrobacterium*-based DNA delivery. Production of fertile, transgenic maize plants was first reported in 1990 using microprojectile bombardment. Reports of fertile transgenic maize plant production using electroporation and silicon carbide fiber treatment followed a few years later.

[0004] The first well-documented report of fertile transgenic maize plants via *Agrobacterium* was published by Ishida et al. in 1996, followed by a second report from Negrotto et al. in 2000. Although high frequency *Agrobacterium*-mediated transformation was reported in those studies, and also in a few maize transformation labs in a private industry, those frequencies have not been reproduced in public maize transformation laboratories. Factors contributing to the lack of reproducibility in the

public sector could include: 1) omission of critical details in protocol and media descriptions in published reports, 2) lack of access to specialized binary vectors by public researchers, and 3) reluctance or inability to transfer proprietary information from private industry to the public sector.

[0005] The significant advantages of using an *Agrobacterium*-based transformation system for maize (high frequency transformation, low copy, simple transgene insertion, increased stability of transgene expression, low cost relative to biolistics, and potential to introduce large DNA fragments into the plant genome) make it imperative that optimized protocols be developed, published, and made readily available to maize researchers in the public sector. Although known for this practical application, the actual mechanism of DNA transfer from bacteria to plants is not completely understood. Moreover, there are some limitations on the use of this transforming vector, e.g. difficulties in transforming monocots, and transforming frequencies may be too low to be useful. At present, even some dicots, for example, many *Arabidopsis* ecotypes and mutants also cannot be easily or efficiently transformed by a root transformation method, generally using *Agrobacterium*.

[0006] It is believed that *Agrobacterium tumefaciens* genetically transforms plant cells by transferring a portion of the bacterial Ti-plasmid, designated the T-DNA, to the plant, and integrating the T-DNA into the plant genome. Little is known about the T-DNA integration process, and no plant genes involved in integration have previously been identified. The DNA that is transferred from *Agrobacterium* to the plant cell is a segment of the Ti, or tumor inducing, plasmid called the T-DNA (transferred DNA). Virulence (vir) genes responsible for T-DNA processing and transfer are reported to lie elsewhere on the Ti plasmid. The role of vir genes in T-DNA processing, the formation of bacterial channels for export of T-DNA, and the attachment of bacteria to the plant cell are reported. In contrast, little is known about the role of plant factors in T-DNA transfer and integration.

[0007] Transformations can be transient or stable. Stable transformation is preferred because it is required to produce transgenic plants.

[0008] Many plant species are recalcitrant to stable *Agrobacterium* transformation. These species are, however, easily transiently transformed to express GUS activity or symptoms of viral infection following agroinoculation. Maize BMS cells are readily transiently transformed and could express and process a *gus-A*-intron

transcript encoded by the binary vector pBISN1. Published results implied that, at least in this transformation system, T-DNA could target to maize nuclei and become converted to a double-stranded transcription-competent form. However, the lack of detectable stable transcription suggested that T-DNA integration may be deficient. Thus, making T-DNA integration more efficient and stabilizing T-DNA gene expression are important factors to improve maize transformation.

[0009] Integration of exogenous DNA is reported to be improved by delivering the DNA into plant cells with one or more *Agrobacterium* genes that can encode for proteins within the plant cells. This technique, referred to as "agrolistic transformation" is just an improvement over biolistic transformation by which DNA is delivered to the plants by a non-biological method such as a "gene gun" (biolistic transformation). In this improvement, genes encoding virulence proteins that normally function in *Agrobacterium* are transferred to the plants along with a T-DNA substrate. The substrate is then acted upon in the plant cell to make a T-DNA molecule. However, the technique described does not include the use of plant genes, or of other factors related herein. The technique was not shown to make a plant more susceptible to transformation. A goal of this method was to increase predictability of the location of integration, not its frequency. Moreover, "agrolistic transformation" is an expensive procedure requiring much infrastructure and resources; one of skill has to go through the laborious process every time to develop a transgenic plant.

[00010] The isolation of a putative plant factor has recently been reported. Ballas and Citovsky showed that a plant karyopherin α (AtKAP α) can interact with VirD2 nuclear localization sequences in a yeast two-hybrid interaction system, and is presumably involved in nuclear translocation of the T-complex. Using a similar approach, a tomato type 2C protein phosphatase, DIG3, that can interact with the VirD2 NLS was identified. Unlike AtKAP α, DIG3 plays a negative role in nuclear import. After the T-DNA/T-complex enters the nucleus, it must integrate into the plant chromosome. Plant chromosomal DNA is packaged into nucleosomes consisting primarily of histone proteins. The incoming T-DNA may have to interact with this nucleosome structure during the integration process. However, T-DNA may preferentially integrate into transcribed regions of the genome. These regions are believed to be temporarily free of histones. How exactly T-DNA integration takes

place is unknown. Recent reports have implicated involvement of VirD2 protein in the T-DNA integration process.

[00011] Several ecotypes of the dicot *Arabidopsis* are resistant to *Agrobacterium* transformation. Transforming the **transformation resistant** *rat5* mutant of *Arabidopsis* with a wild-type *RAT5* (histone H2A) gene was reported by the inventors to complement the mutant phenotype.

[00012] In monocots, maize is the most studied model plant that has important economic value. Although genetic transformation systems for the maize have been established in private laboratories, the lack of such systems is still a key limitation for public researchers. This is because most public research groups do not have access to the resources and infrastructure necessary for maize transformation by currently available procedures. In addition, the current technology has serious limitations, including low efficiency and throughput, difficulty with inbred line transformation, unpredictable transgene copy numbers and integrity, and undesirable transgene silencing during development and over generations.

[00013] Because fertile transgenic maize (*Zea mays*) was first produced using the biolistic gun, maize transformation technology has served as an important tool in germplasm development and research addressing fundamental biological questions through the study of transgenic maize. Recent reports have demonstrated that *Agrobacterium tumefaciens*-mediated maize transformation may offer a better alternative than the biolistic gun for delivery of transgenes to maize. This gene delivery system results in a greater proportion of stable, low-copy number transgenic events than does the biolistic gun, offers the possibility of transferring larger DNA segments into recipient cells, and is highly efficient. Reproducible protocols for *A. tumefaciens*-mediated maize transformation have used super binary vectors, in which the *A. tumefaciens* strain carries extra copies of *virB, virC*, and *virG*, to infect immature zygotic embryos of the inbred line A188 or the hybrid line Hi II. Hi II immature zygotic embryos were transformed by the inventor at an average efficiency of 5.8% using the *A. tumefaciens* super binary vector in strain LBA4404. Because the cost of licensing this proprietary technology for use on a broader scale may be prohibitive to a public sector laboratory, the inventors implemented an *A. tumefaciens* standard binary (non-super binary) vector system to transform maize Hi II immature zygotic embryos. Stable transformation of maize using a standard binary vector to infect shoot meristems was

reported previously, but adoption of this method was hindered by its lack of robustness. Development of a reproducible and efficient method for transforming maize using a standard binary vector will not only provide researchers with the benefits already outlined, it would also facilitate vector construction when compared with the super binary vector. Final assembly of a super binary vector system involves co-integration of the gene of interest into a large plasmid (pSB1) in *A. tumefaciens* strain LBA4404 via homologous recombination. In contrast, assembly of a standard binary vector does not require this additional step, making it a more efficient way to confirm the introduction of a gene of interest into an *A. tumefaciens* strain.

[00014] Transformation of maize (*Zea mays*) using an *Agrobacterium tumefaciens* standard binary (non-super binary) vector system was achieved by the inventors. Immature zygotic embryos of the hybrid line Hi II were infected with *A. tumefaciens* strain EHA101 harboring a standard binary vector and cocultivated in the presence of 400 mg/L L-cysteine. Inclusion of L-cysteine in cocultivation medium led an improvement in transient -glucuronidase expression observed in targeted cells and a significant increase in stable transformation efficiency, but was associated with a decrease in embryo response after cocultivation. The average stable transformation efficiency (no. of bialaphos-resistant events recovered per 100 embryos infected) was 5.5%. Southern-blot and progeny analyses confirmed the integration, expression, and inheritance of the bar and gus transgenes in R_0, R_1, and R_2 generations of transgenic events. Fertile, stable transgenic maize was routinely produced using an *A. tumefaciens* standard binary vector system.

[00015] The level of stable transformation achieved is attributed to supplementation of cocultivation medium with 400 mg/L Cys. This antioxidant treatment also increased T-DNA delivery to embryogenic-competent scutellum cells of infected embryos. A similar increase in transient gus gene expression, followed by an increase in stable transformation efficiency, was reported in soybean cotyledonary node explants infected with *A. tumefaciens* and cocultivated on medium supplemented with Cys.

[00016] Contrary to expectations, the increase in stable transformation efficiency observed with the 400 mg/L Cys treatment was associated with a decrease in the proportion of embryos giving rise to embryogenic callus compared with the 0 mg/L Cys treatment. This reduction in embryo response is not related to the plant-pathogen interaction per se because noninfected embryos also exhibited reduced response on 400 mg/L Cys. It

is likely that Cys concentrations as high as 400 mg/L are toxic to maize cells. A similar negative impact of 80 mg/L Cys on embryogenesis in Japonica rice explants was reported by Enriquez-Obregon et al. (1999). Comparable stable transformation rates were achieved using Cys concentrations as low as 100 mg/L, and this treatment was associated with better embryo recovery after cocultivation than that observed using the 400 mg/L Cys treatment.

[00017] *A. tumefaciens*-mediated maize transformation using a standard binary vector system is reproducible although variability in experimental efficiency persists. Using cocultivation medium within 7 d of preparation minimizes this variability. Average transformation efficiency is about 5.5%.

[00018] Information on plant factors and other factors affecting *Agrobacterium* transformation frequencies in plants is needed to improve performance of this method in both dicots and monocots.

SUMMARY OF THE INVENTION

[00019] Methods and compositions for increasing *Agrobacterium* transformation efficiency in a host plant include adding histones to the host plant and, for monocots, also adding L-cysteine and using a standard (a non-super, "Simple Binary Vector System") binary vector. Histones may be added either transiently or genes encoding histone may be stably incorporated into a host plant genome.

[00020] A polynucleotide sequence encoding a plant histone protein may be integrated into the host plant genome or just transiently introduced to express the polynucleotide sequence encoding a plant histone protein.

[00021] The host plant expressing the polynucleotide sequence encoding a plant histone protein to increase base levels of histone is transformed with a DNA molecule of interest by means of *Agrobacterium*.

[00022] There are four classes of core histones (H2A, H2B, H3 and H4). A suitable plant histone protein is a member of an H2A gene family of *Arabidopsis*, for example *RAT5*.

[00023] An aspect of the invention is a transgenic plant with at least one additional copy of a polynucleotide sequence encoding a plant histone H2A protein. The polynucleotide sequence may encode a plant histone H2A protein that is a member of an H2A gene family of *Arabidopsis*.

[00024] A method for increasing stable *Agrobacterium* transformation efficiency in host plants, includes the steps of:

(a) selecting a host plant expressing a polynucleotide sequence encoding a plant histone H2A protein, thereby increasing levels of histone in the host plant;

(b) introducing a transformation vector with a DNA molecule of interest into an *Agrobacterium* strain;

(c) providing at least one antioxidant in a cocultivation medium;

(d) infecting cells from the host plant with the *Agrobacterium* strain in the cocultivation medium;

(e) providing conditions suitable for recovery of infected cells; and

(f) selecting the infected cells for transformants expressing the DNA molecule of interest.

[00025] The host plant may be a monocot, for example, maize.

[00026] The antioxidant may be L-cysteine.

[00027] The L-cysteine concentration may be between about 100 mg/L and 400 mg/L.

[00028] Infection of cells in the cocultivation medium may be for 3 days.

[00029] An aspect of the invention is a genetic construct comprising at least one copy of a histone gene in addition to that in the host plant initially that when expressed is capable of increasing transformation frequencies in a host plant.

[00030] The histone gene may encode H2A, for example a RAT5 *Arabidopsis* gene.

[00031] An aspect of the invention is a host cell transformed by at least one copy of a gene involved in T-cell integration wherein the gene is capable of effecting overexpression of histone to enhance plant transformation frequencies.

[00032] A method for increasing *Agrobacterium* transformation frequencies in a host plant, includes the steps of:

a. increasing histone levels in the host plant compared to normal levels of histone in the host plant; and

b. transforming the host plant with *Agrobacterium*.

[00033] Transformation frequencies may be measured by the number of tumors produced in the host plant or by using markers detectable if transformation has occurred.

[00034] The H2A histone may be H2A-1.

[00035] An aspect of the invention is a plant cell with an overexpression of plant histones sufficient to increase efficiency of transformation of the plant cell by *Agrobacterium*.

Definitions

[00036] **Transformation efficiency**: (no. of successful events/no. of embryos infected) X 100. The number of transgenic events is a indication of stable transformation. The transformation efficiency calculation may also involve inflorescences, callus, seeds or other biological material that can be infected with *Agrobacterium* to produce a transformant.

[00037] **H2A**: A member of H2A gene family. The H2A gene members are also denoted by HTA. Arabidopsis RAT5 is one such member of H2A/HTA gene family.

[00038] **Retransformation**: transformation of a host plant that has at least one additional copy of a polynucleotide encoding histone H2A protein stably integrated into the host plant genome.

[00039] **Infected**: *Agrobacterium* is in the host cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[00040] FIG. 1 shows characteristics of the *rat5* mutant: (A) stable transformation of wild-type *Arabidopsis* ecotype Ws, the *rat5* mutant, and the F1 progeny; (B) sequence of the *rat5*/T-DNA junction region; (C) pattern of T-DNA integration in *rat5*: LB, T-DNA left border; RB, T-DNA right border; pBR322, pBR322 sequences containing the β-lactamase gene and ColE1 origin of replication; Tn903, kanamycin resistance gene for *E. coli* selection; Tn5, kanamycin resistance gene for plant selection.

[00041] FIG. 2 shows complementation of the *rat5* mutant and overexpression of *RAT5* in wild-type *Arabidopsis* plants; (A) maps of the binary vectors pKM4 and (B) pKM5 RB, T-DNA right border; LB, T-DNA left border; pAnos, nopaline synthase polyadenylation signal sequence; histone H2A, coding sequence of the *RAT5* histone H2A gene; pH2A, promoter sequence of the *RAT5* histone H2A gene; Pnos, nopaline synthase promoter; hpt, hygromycin resistance gene; pAg7, agropine synthase polyadenylation signal sequence; uidA, promoterless gusA gene; arrows above the histone H2A, uidA, and hpt genes indicate the direction of transcription; (C) complementation of the *rat5* mutant; (D) tumorigenesis assay of Ws transgenic plants overexpressing the *RAT5* histone H2A gene.

[00042] FIG. 3 shows T-DNA integration assays of *rat5* and Ws plants; (A) transient and stable GUS expression in Ws and *rat5*; (B) T-DNA integration in *rat5* and Ws plants.

[00043] FIG. 4 shows the map of the binary maize transformation vector pE2250 to generate the founder lines of maize and a flow chart of the construction of the vector.

[00044] FIG. 5 shows a northern blot of H2A expressing transgenic maize lines.

[00045] FIG. 6(A-E) shows the cDNA sequences of HTA1 through HTA13. The coding region is highlighted in bold font and 5' and 3' UTR region in normal font.

[00046] FIG. 7 (A-B) shows the amino acid sequences of HTA1 through HTA13.

[00047] FIG. 8 (A-M) shows the genomic sequences of HTA1 through HTA13.

[00048] FIG. 9 shows that *Agrobacterium*-mediated transformation results in lower transgene copy number (A) and higher gene expression (B) compared with biolistic gun transformation of maize (Copy number: L: 1-3; M: 4-10; H: 10-20; VH: >20). (C-D) show that transgene expression is more stable in *Agrobacterium*-derived transformants (C) than in bombardment-derived ones (D).

[00049] FIG. 10 is a schematic illustration of a T-DNA region of a standard binary vector pTF102. LB, Left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, -glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; H, HindIII.

DETAILED DESCRIPTION OF THE INVENTION

[00050] The invention relates to methods and composition to increase *Agrobacterium* transformation frequencies in plants due to addition of histones directly or by incorporating into the host plant at least one plant gene involved in host T-DNA integration. This differs from some methods in prior publications because plant host genes, not bacterial *Agrobacterium* genes, are used to enhance transformation. In an embodiment, addition of at least one histone H2A gene encoded by the *Arabidopsis RAT5* gene enhances transformation frequencies, most likely due to overexpressing of histone as compared to the host's natural expression levels. The gene can be either in transgenic plants or carried by the transforming agent, T-DNA, for practice of the invention.

[00051] To identify plant genes involved in *Agrobacterium*-mediated transformation, a T-DNA tagged *Arabidopsis* library was screened for mutants that are resistant to *Agrobacterium* transformation (*rat* mutants). An *Arabidopsis* T-DNA tagged mutant, *rat5*, was characterized that is deficient in T-DNA integration and is resistant to *Agrobacterium*-mediated root transformation. Both genetic and DNA blot analyses indicated that there are two copies of T-DNA integrated as a tandem repeat at a single locus in *rat5*. No major rearrangements are in the *rat5* plant DNA immediately

surrounding the T-DNA insertion site. These data strongly suggest that in *rat5* the T-DNA had inserted into a gene necessary for *Agrobacterium*-mediated transformation. The sequence of the T-DNA left border-plant junction indicated that the T-DNA had inserted into the 3' untranslated region of a histone H2A gene. This insertion is upstream of the consensus polyadenylation signal. By screening an *Arabidopsis* ecotype Ws cDNA library and sequencing 20 different histone H2A cDNA clones, and by performing a computer data base search, at least six different histone H2A genes were identified. These genes encode proteins that are greater than 90% identical at the amino acid sequence level. Thus, the histone H2A genes comprise a multi-gene family in *Arabidopsis*.

[00052] The gene bank accession number AB016879 contains a report of a DNA sequence of some of the clones derived from *Arabidopsis thaliana* chromosome 5. One of these sequences is a histone H2A gene that is identical to the *RAT5* gene. However, this report neither teaches nor suggests a role for histone in improving transformation frequencies.

[00053] Overexpression of histone genes of the present invention overcomes the poor performance that limits the use of *Agrobacterium* as a transforming vector. Many plants can be transformed transiently by *Agrobacterium* so they express the transforming gene for a period of time, but are not stably transformed because of T-DNA integration problems. Therefore, transgenic plants are not produced. The gene H2A (*RAT5*) plays an important role in illegitimate recombination of T-DNA into the plant genome and the gene's overexpression enhances transformation.

[00054] Transient and stable GUS (β-glucuronidase) expression data and the assessment of the amount of T-DNA integrated into the genomes of wild-type and *rat5 Arabidopsis* plants indicated that the *rat5* mutant is deficient in T-DNA integration needed for transformation. Complementing the *rat5* mutation was accomplished by expressing the wild-type *RAT5* histone H2A gene in the mutant plant. Surprisingly, overexpression of *RAT5* in wild-type plants increased *Agrobacterium* transformation efficiency. Furthermore, transient expression of a *RAT5* gene from the incoming T-DNA was sufficient to complement the *rat5* mutant and to increase the transformation efficiency of wild-type *Arabidopsis* plants. Adding histone directly to the host plant enhances transformation. The present invention provides methods and compositions

to increase stable transformation frequency in plants using direct involvement of a plant histone gene in T-DNA integration.

[00055] Several T-DNA tagged mutants of *Arabidopsis* were identified that are recalcitrant to *Agrobacterium* root transformation. These are called rat mutants (resistant to *Agrobacterium* transformation). In most of these mutants *Agrobacterium* transformation is blocked at an early step, either during bacterial attachment to the plant cell or prior to T-DNA nuclear import. In some of the mutants, however, the T-DNA integration step is most likely blocked. Because plant factors involved in illegitimate recombination of T-DNA into the plant genome have not previously been identified, the characterization of a T-DNA tagged *Arabidopsis* mutant, *rat5*, that is deficient in T-DNA integration, is an aspect of the present invention.

[00056] **Characterization of the *rat5* mutant.** *rat5*, an *Arabidopsis* T-DNA tagged mutant, was previously identified as resistant to *Agrobacterium* root transformation. *rat5* mutants are also expected in other species, e.g. maize. An in vitro root inoculation assay was performed using the wild-type *Agrobacterium* strain A208 (At10). After one month, the percentage of root bundles that formed tumors was calculated. Greater than 90% of the root bundles of the wild-type plants (ecotype Ws) formed large green teratomas. In contrast, fewer than 10% of the root bundles from the *rat5* plants responded to infection, forming small yellow calli (FIG. 1A). A homozygous *rat5* plant (pollen donor) was crossed to a wild-type plant (egg donor) and the resulting F1 progeny tested for susceptibility to *Agrobacterium* transformation. This analysis indicated that *rat5* is a dominant mutation (FIG. 1A). Further analysis of F2 progeny indicated that kanamycin resistance segregated 3:1, indicating that a single locus had been disrupted by the mutagenizing T-DNA. Kanamycin resistance co-segregated with the *rat5* phenotype, indicating that a gene involved in *Agrobacterium* transformation had most likely been mutated by the T-DNA insertion.

[00057] **Recovery of a T-DNA-plant junction from *rat5*.** The T-DNA integration pattern in the *rat5* mutant was determined by DNA blot analyses. The results indicated that there are only two copies of the mutagenizing T-DNA integrated into the genome of the *rat5* mutant. Further analysis indicated that these two T-DNA copies are present as a direct tandem repeat, as shown in FIG. 1C.

[00058] A left border (LB) T-DNA-plant junction was recovered from *rat5* using a plasmid rescue technique (see Materials and Methods) and a restriction endonuclease map of this T-DNA-plant junction was constructed. An approximately 1.7 kbp EcoRI fragment that contains both plant and LB DNA was subcloned into pBluescript and subsequently sequenced at the Purdue University sequencing center. The sequence of this fragment is shown in FIG. 1B. DNA sequence analysis of this junction region indicated that the T-DNA had inserted into the 3' untranslated region (UTR) of a histone H2A gene (FIG. 1B). The histone H2A genes of *Arabidopsis* were further characterized by isolating and sequencing numerous cDNA and genomic clones. Six different gene variants of histone H2A were identified, indicating that the histone H2A genes of *Arabidopsis* comprise a small multi-gene family. In a lambda genomic DNA library a clone was identified containing the wild-type histone H2A gene corresponding to *RAT5*. DNA sequence analysis of this genomic clone indicated that in *rat5* the T-DNA had inserted upstream of the consensus polyadenylation signal (AATAA). DNA blot analysis of Ws and *rat5* DNA indicated that the T-DNA insertion in *rat5* did not cause any major rearrangements in the plant DNA immediately around the site of insertion. Disruption of the 3' UTR of the *RAT5* histone H2A gene is likely the sole cause for the rat phenotype in the *rat5* mutant.

[00059] FIG. 1 shows characterization of the *rat5* mutant. (A) Stable transformation of wild-type *Arabidopsis* ecotype Ws, the *rat5* mutant, and the F1 progeny. Sterile root segments were infected with A. tumefaciens A208. Two days after cocultivation, the roots were transferred to MS medium lacking phytohormones and containing timentin as an antibiotic. Tumors were scored after four weeks. (B) Sequence of the *rat5*/T-DNA junction region. (C) Pattern of T-DNA integration in *rat5*. LB, T-DNA left border; RB, T-DNA right border; pBR322, pBR322 sequences containing the β-lactamase gene and ColE1 origin of replication; Tn903, kanamycin resistance gene for *E. coli* selection; Tn5, kanamycin resistance gene for plant selection. Five μg of genomic DNA from the *rat5* mutant was digested with either EcoRI or SalI and was blotted onto a nylon membrane. An EcoRI-SalI fragment of pBR322 was used as the hybridization probe. Restriction fragment sizes shown above the T-DNA were detected by EcoRI digestion and the sizes shown below the T-DNA were detected by SalI digestion.

[00060] **Complementation of the *rat5* mutant with a wild-type histone H2A gene (*RAT5*).** Two different constructions were made to perform a complementation analysis of the *rat5* mutant. First, a nopaline synthase terminator (3' NOS) was fused to the 3' region of the 1.7 kbp junction fragment (the sequence of this 1.7 kbp fragment is shown in FIG. 1B). This construction contains the *RAT5* histone H2A gene with its own promoter and a 3' NOS. This fragment (*RAT5* plus 3' NOS) was cloned into the binary vector pGTV-HPT of beaker containing a hygromycin resistance gene between the left and the right T-DNA borders, resulting in the binary vector pKM4 (FIG. 2A). For the second construction, a 9.0 kbp SacI genomic fragment of wild-type Ws DNA containing a histone H2A gene (*RAT5*) plus at least 2.0 kbp sequences upstream and downstream of *RAT5* was cloned into the binary vector pGTV-HPT, resulting in the binary vector pKM5 (FIG. 2B). pKM4 and pKM5 were transferred separately into the non-tumorigenic *Agrobacterium* strain GV3101, resulting in strains A. tumefaciens At1012 and At1062, respectively.

[00061] Both strains At1012 and At1062 were separately used to transform *rat5* plants using a germ-line transformation method (Bent *et al.*, 1998) and transgenic *rat5* plants were selected for resistance to hygromicin (20 μg/ml). Several transgenic plants (T1) were obtained. These transgenic plants were allowed to self fertilize and T1 seeds were collected. Six transgenic lines obtained by transformation with At1012 (the wild-type histone H2A with 3' NOS) were randomly selected and their seeds were germinated in the presence of hygromycin. Tumorigenesis assays were performed as described in Nam *et al.* (1999) using A. tumefaciens At10 and a sterile root inoculation protocol, on at least five different plants from each of the six transgenic lines. The results indicated that in five of the six transgenic *rat5* lines tested, the tumorigenesis-susceptibility phenotype was recovered (FIG. 2C; Table 1). Teratomas incited on the roots of these plants appeared similar to tumors generated on a wild-type plant. One of the transgenic plants tested did not recover the tumorigenesis-susceptibility phenotype, probably because of an inactive transgene. Transgenic T1 plants of *rat5* obtained by transformation with At1062 (containing a genomic encoding *RAT5* from the wild-type plant) were also tested for restoration of the tumorigenesis-susceptibility phenotype. Some of these plants were also able to recover the tumorigenesis-susceptibility phenotype, indicating complementation of the *rat5* mutation. Hygromycin-resistant transgenic plants generated by transforming

the *rat5* mutant with pGPTV-HPT alone did not form tumors upon infection with A. tumefaciens A208.

[00062] To confirm the genetic basis of the complementation experiment, a co-segregation analysis was performed on one of the *rat5* transgenic lines (*rat5* At1012-6) obtained by transformation of the *rat5* mutant with *A. tumefaciens* At1012. To examine the co-segregation of the complementing T-DNA containing the wild-type *RAT5* gene with the tumorigenesis-susceptibility phenotype, seeds from a T2 plant homozygous for the *rat5* mutation but heterozygous for hygromycin resistance were germinated and grown on B5 medium without selection. Roots of these plants were subsequently tested for hygromycin-resistance and susceptibility to crown gall tumorigenesis. All plants that were sensitive to hygromycin were also resistant to tumor formation in a manner similar to that of the *rat5* mutant. Of the 25 hygromycin-resistant plants, at least 8 were susceptible to tumorigenesis. However, 17 hygromycin-resistant plants remained recalcitrant to *Agrobacterium*-mediated transformation. It is likely that these plants are heterozygous with respect to the complementing *RAT5* gene and did not express this gene to a level high enough to restore susceptibility to tumorigenesis. This possibility corresponds to the finding that the *rat5* mutation is dominant, and that therefore one active copy of *RAT5* is not sufficient to permit *Agrobacterium*-mediated transformation. Taken together, the molecular and genetic data strongly indicate that in the *rat5* mutant disruption of a histone H2A gene is responsible for the tumorigenesis-deficiency (rat) phenotype.

[00063] Overexpression of a histone H2A (*RAT5*) gene in wild-type plants improves the efficiency of *Agrobacterium* transformation. To determine further whether the *RAT5* gene plays a direct role in *Agrobacterium*-mediated transformation, *A. tumefaciens* At1012 was used to generate several transgenic *Arabidopsis* plants (ecotype Ws) containing additional copies of the *RAT5* histone H2A gene. These transgenic plants were allowed to self-pollinate, T1 seeds were collected, and T2 plants were germinated in the presence of hygromycin. Tumorigenesis assays were performed as described herein at least five plants from each of four different transgenic lines. Because ecotype Ws normally is highly susceptible to *Agrobacterium* transformation, the tumorigenesis assay was altered to detect any subtle differences between the transformation-susceptible wild-type plant and transgenic wild-type plants overexpressing *RAT5*. These alterations included inoculation of root segments with a

100-fold lower concentration (2x107 cfu/ml) of bacteria than that normally used (2x109 cfu/ml), and spreading individual root segments rather than bundles of root segments on MS medium to observe tumor production. The results, shown in Table 1 and FIG. 2D, indicate that transgenic plants overexpressing *RAT5* are approximately twice as susceptible to root transformation as are wild-type Ws plants. These data indicate that the *RAT5* histone H2A gene plays a direct role in T-DNA transformation, and that overexpression of *RAT5* can increase susceptibility to transformation.

[00064] Transient expression of histone H2A is sufficient to permit transformation of *rat5* and to increase the transformation efficiency of wild-type Ws plants. Expression of the *RAT5* histone H2A gene from the incoming T-DNA complement the *rat5* mutant. Although transformation of this mutant with an *Agrobacterium* strain harboring pGPTV-HYG (lacking a histone H2A gene) resulted in only a few, slow-growing calli on hygromycin selection medium, *Agrobacterium* strains harboring pKM4 or pKM5 incited rapidly growing hygromycin-resistant calli on 60±21% and 54±22% of the *rat5* root segment bundles, respectively. In addition, when wild-type plants were infected (at low bacterial density) with a tumorigenic *Agrobacterium* strain (A208) harboring pKM4, 78±8% of the root segments developed tumors, compared to 36±9% of the root segments infected with a tumorigenic bacterial strain harboring pGPTV-HYG. These transformation experiments indicate that *Agrobacterium* strains containing the binary vectors pKM4 or pKM5 are able to transform *rat5* mutant plants at relatively high efficiency, and on wild-type plants are two-fold more tumorigenic, and are better able to incite hygromycin-resistant calli, than are *Agrobacterium* strains containing the "empty" binary vector pGPTV-HYG. Transiently produced histone H2A improves the stable transformation efficiency of plants by *Agrobacterium*.

[00065] The *rat5* mutant is deficient in T-DNA integration. *Agrobacterium*-mediated transformation of the *Arabidopsis rat5* mutant results in a high efficiency of transient transformation but a low efficiency of stable transformation, as determined by the expression of a gusA gene encoded by the T-DNA. This result suggested that *rat5* is most likely deficient in T-DNA integration. To test this hypothesis directly root segments from Ws and *rat5* plants were inoculated with *A. tumefaciens* GV3101 harboring the T-DNA binary vector pBISN1. pBISN1 contains a gusA-intron gene under the control of a "super-promoter" (Ni *et al.*, 1995; Narasimhulu *et al.*, 1996).

Two days after cocultivation, the root segments were transferred to callus inducing medium containing timentin (100 μg/ml) to kill the bacteria. Three days after infection, a few segments were stained for GUS activity using the chromogenic dye X-gluc. Both the wild-type and the *rat5* mutant showed high levels of GUS expression (approximately 90% of the root segments stained blue; FIG. 3A). The remaining root segments were allowed to form calli on callus inducing medium containing timentin to kill *Agrobacterium*, but lacking any antibiotic for selection of plant transformation. After four weeks numerous calli derived from at least five different Ws and *rat5* plants were stained with X-gluc. Of the Ws calli sampled, 92±12% showed large blue staining areas, whereas only 26±10% of the *rat5* calli showed GUS activity, and most of these blue staining regions were small (FIG. 3A). These data indicate that although the *rat5* mutant can transiently express the gusA gene at high levels, it fails to stabilize gusA expression.

[00066] Suspension cell lines were generated from these Ws and *rat5* calli and after an additional month the amount of T-DNA was assayed (using as a hybridization probe the gusA-intron gene located within the T-DNA of pBISN1) integrated into high molecular weight plant DNA from Ws and *rat5* calli (Nam *et al.*, 1997; Mysore *et al.*, 1998). FIG. 3B shows that although T-DNA integrated into the genome of wild-type Ws plants was easily detectable, T-DNA integrated into the *rat5* genome was not. These data directly demonstrate that *rat5* is deficient in T-DNA integration. To demonstrate equal loading of plant DNA in each of the lanes, the gusA probe was stripped from the blot and rehybridized the blot with an *Arabidopsis* phenylalanine ammonia-lyase (PAL) gene probe.

[00067] FIG. 2 shows complementation of the *rat5* mutant and overexpression of *RAT5* in wild-type *Arabidopsis* plants. Maps of the binary vectors pKM4 (A) and pKM5 (B). RB, T-DNA right border; LB, T-DNA left border; pAnos, nopaline synthase polyadenylation signal sequence; histone H2A, coding sequence of the *RAT5* histone H2A gene; pH2A, promoter sequence of the *RAT5* histone H2A gene; Pnos, nopaline synthase promoter; hpt, hygromycin resistance gene; pAg7, agropine synthase polyadenylation signal sequence; uidA, promoterless gusA gene. Arrows above the histone H2A, uidA, and hpt genes indicate the direction of transcription. (C) Complementation of the *rat5* mutant. *rat5* mutant plants were transformed with an *Agrobacterium* strain containing the binary vector pKM4 (At1012). Hygromycin-

resistant transgenic plants were obtained and were self-pollinated to obtain T2 plants. Sterile root segments of T2 plants expressing *RAT5*, wild-type Ws plants, and *rat5* mutant plants were infected with the tumorigenic strain *A. tumefaciens* A208. Two days after cocultivation, the roots were moved to MS medium lacking phytohormones and containing timentin. Tumors were scored after four weeks. (D) Tumorigenesis assay of Ws transgenic plants overexpressing the *RAT5* histone H2A gene. Ws plants were transformed with *A. tumefaciens* At1012 containing the binary vector pKM4. Hygromycin-resistant transgenic plants were obtained and were self-pollinated to obtain T2 plants. Sterile root segments of T2 plants overexpressing *RAT5* and wild-type Ws plants were infected at low bacterial density with *A. tumefaciens* A208. After two days cocultivation, the roots were moved to MS medium lacking phytohormones and containing timentin. Tumors were scored after four weeks.

[00068] Teratomas incited on the roots of these plants appeared similar to tumors generated on a wild-type plant. One of the transgenic plants tested did not recover the tumorigenesis-susceptibility phenotype, probably because of an inactive transgene. Transgenic T1 plants of *rat5* obtained by transformation with At1062 (containing a genomic encoding *RAT5* from the wild-type plant) were also tested for restoration of the tumorigenesis-susceptibility phenotype. Some of these plants were also able to recover the tumorigenesis-susceptibility phenotype, indicating complementation of the *rat5* mutation. Hygromycin-resistant transgenic plants generated by transforming the *rat5* mutant with pGPTV-HPT alone did not form tumors upon infection with *A. tumefaciens* A208.

[00069] FIG. 3 shows T-DNA integration assays of *rat5* and Ws plants; (A) transient and stable GUS expression in Ws and *rat5*; Sterile root segments of Ws and *rat5* plants were infected with the non-tumorigenic *Agrobacterium* strain GV3101 containing the binary vector pBISN1. Two days after cocultivation, the roots were transferred to callus inducing medium (CIM) containing timentin. Three days after infection, half of the segments were stained with X-gluc to determine the efficiency of transient GUS expression. The other group of segments was allowed to form calli on CIM. After four weeks these calli were stained with X-gluc to determine the efficiency of stable GUS expression. (B) T-DNA integration in *rat5* and Ws plants. Suspension cells were derived from the calli generated from Ws and *rat5* root segments infected with the non-tumorigenic *Agrobacterium* strain GV3101 containing the binary vector

pBISN1. The suspension cell lines were grown for three weeks (without selection for transformation) in the presence of timentin or cefotaxime to kill *Agrobacterium*. Genomic DNA was isolated from these cells, subjected to electrophoresis through a 0.6% agarose gel, blotted onto a nylon membrane, and hybridized with a gusA gene probe. After autoradiography, the membrane was stripped and rehybridized with a phenylalanine ammonia-lyase (PAL) gene probe to determine equal loading of DNA in each lane.

[00070] **Table 1.** Complementation of the *rat5* mutant and overexpression of *RAT5* in wild-type (Ws) *Arabidopsis* plants

Line	% Root Bundles With Tumors	Tumor Morphology
rat5 complementation with At1012 (T2 plants)[a]		
Ws	98±2	large, green
rat5	21±6	small, yellow
rat5 At1012-1	64±30	large + small, green
rat5 At1012-2	17±4	small, yellow
rat5 At1012-3	70±20	large + medium, green
rat5 At1012-4	86±6	large, green
rat5 At1012-5	82±10	large, green
rat5 At1012-6	92±5	large, green

[00071] Overexpression of *RAT5* in Ws (T2 plants)[ab]

Ws	35±14	large, green
Ws At1012-1	69±27	large, green
Ws At1012-2	68±25	large, green
Ws At1012-3	64±13	large, green
Ws At1012-4	63±20	large, green

[a] at least 5 plants were tested for each mutant and 40-50 root bundles were tested for each plant
[b] *Agrobacterium* was diluted to a concentration 100-fold lower than that normally used, and single root segments were separated

EXAMPLES

Example 1: Results Indicating the Value of Using the *Arabidopsis* histone H2A-1 Gene to Improve Plant Transformation

[00072] Evidence from two independent lines of experimentation shows that the *Arabidopsis* histone H2A-1 is useful to improve the efficiency of *Agrobacterium*-mediated plant transformation in dicots and monocots.

[00073] 1. Many *Arabidopsis* ecotypes and mutants cannot be easily transformed by a root transformation method (although they can still be transformed by the flower-dip method). The flower-dip method was used to introduce a histone H2A-1 cDNA, under the control of the CaMV 35S promoter, into a large number of recalcitrant ecotypes and mutants. A number of these transgenic lines were analyzed and evidence emerged that all ecotypes/mutants tested to date can be made competent for root transformation when they over-express the H2A-1 gene. These include mutants in the *Agrobacterium* attachment process (*rat1* and *rat3*), T-DNA integration (*rat17, rat18, rat20,* and *rat22*), a chromatin mutant (*HAT6*), and several other mutants with as yet uncharacterized lesions (*rat21* and *ratJ7*). Additionally, several recalcitrant ecotypes can be made more susceptible to transformation when the H2A-1 cDNA is over-expressed. These include the ecotypes Ag-0 and Dijon-G.

[00074] Other suitable mutants include (*rat4, rat14, rat15, ratJ1, α7, T9* and *T16*) and ecotypes (*Cal-0, UE-1, Ang-0, Petergof,* and *BI-1*) when over-expressing the H2A-1 gene may also be more susceptible to *Agrobacterium*-mediated transformation.

[00075] 2. Kan Wang at the Plant Transformation Facility, Iowa State University tested for the present invention in two different *Agrobacterium* strains. One contains a T-DNA binary vector with a herbicide resistance gene in the T-DNA (this is the control construction)l. The other strain contains a similar T-DAN binary vector, bit in addition to the herbicide resistance gene the T-DNA contains the *Arabidopsis* histone H2A-1 cDNA under the control of maize adh1 promoter and intron. These strains were used in four rounds of maize transformation experiments. Usually, transformation and regeneration of maize requires an anti-oxidant (such as L-cysteine) to prevent tissue browning and necrosis as a response to the bacteria. Several thousand transformations (using the control vector without the histone gene) produced virtually no transformants. In these experiments, there were no transformants (using the control strain) without L-cysteine. With L-cysteine, about 2-3% of the infected immature embryos give transformants. Using the histone gene and L-cysteine, there

was 2-3% transformation. However, with the histone gene and without L-cysteine, they obtained 2 (0.2%) transformants. Preliminary results suggest that the histone gene may sensitize the maize embryos to transformation so that a few transformants can be obtained in the absence of an anti-oxidant.

Example 2: Improved Agrobacterium-based transformation of monocot plants with an H2A gene.

Embryos from wild -type maize plants were transformed with *Agrobacterium* containing a histone H2A gene and an antibiotic resistance gene in the presence of L-cysteine in the cocultivation medium. Transgenic T1 plants (founder lines) were obtained and their seeds (T2) were collected. T2 plants were selected on antibiotic resistance growth medium and based on histone H2A RNA expression data. Embryos from selected T2 plants (T2 embryos) were retransformed with *Agrobacterium* containing a standard binary vector and a gene of interest in the presence of L-cysteine in the cocultivation medium. An increase in the number of embryos responding indicated an increase in transformation efficiency over transformation using histone alone. Table 2 shows the overall efficiency of transformation [total putatives by event/total responding by event].

Agrobacterium-mediated transformation results in lower transgene copy number and higher gene expression compared with biolistic gun transformation of maize (FIG. 9 A-B). Transgene expression is also more stable in *Agrobacterium*-derived transformants than in bombardment-derived ones (FIG. 9C-D).

[00076] Total RNA was extracted from leaves of transgenic plants twice independently, and duplicate loadings were run per extract. Thus there were four data points for each sampled plant. Plant number 9 was incorrectly identified as transgenic; it had no expression of the H2A-1 construction and therefore was used as a background control.

[00077] Overall the H2A-1 gene was more highly expressed in A10 (H2A-containing) than in A17 (H2A lacking) lines (FIG. 5). Expression levels among the individual plants differed considerably within the A10 and A17 groups. Plant lane 2 was most intensely hybridizing, followed by the group of lines 5, 8, 6 and 7 (average), which were similar to one another (FIG. 5). The remaining lanes were less intensely hybridizing.

MATERIALS AND METHODS

[00078] **Nucleic acid manipulation.** Total plant genomic DNA was isolated according to the method of Dellaporta *et al.* (1983). Restriction endonuclease digestions, agarose gel electrophoresis, plasmid isolation, and DNA blot analysis were conducted as described (Sambrook *et al.*, 1982).

[00079] **Plasmid Rescue.** Genomic DNA (5 μg) of *rat5* was digested to completion with SalI. The digested DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA was self-ligated in a final volume of 500 μl in 1 x ligation buffer (Promega) with 3 units of T4 DNA ligase at 16oC for 16 hr. The ligation mixture was precipitated with ethanol, transformed into electrocompetent E. coli DH10B cells (mcrBC-; Life Technologies, Inc., Gaithersburg, MD) by electroporation (25 μF, 200 Ω, and 2.5 kV) and plated on LB medium containing ampicillin (100 μg/ml). Ampicillin-resistant colonies were lifted onto a nylon membrane, the bacteria were lysed, and DNA was denatured in situ (Sambrook *et al.*, 1982). A radiolabeled left border (LB) sequence (3.0 kbp EcoRI fragment of pE1461) was used as a hybridization probe to identify a plasmid containing the LB. Positive colonies were picked and plasmid DNA was isolated. By restriction fragment analysis a plasmid containing both the LB and plant junction DNA was identified. The plant junction fragment was confirmed by hybridizing the junction fragment to wild-type plant DNA. A restriction map of this plasmid, containing the LB-plant junction DNA, was made. A 1.7 kbp EcoRI fragment that contained plant DNA plus 75 base pairs of LB sequence was subcloned into pBluescript, resulting in pE1509. This fragment was subsequently sequenced at the Purdue University sequencing center.

[00080] Growth of *Agrobacterium* and in vitro root inoculation of *Arabidopsis* thaliana. These were performed as described previously by Nam *et al.* (1997).

[00081] **Plant Growth Conditions.** Seeds of various *Arabidopsis* thaliana ecotypes were obtained from S. Leisner and E. Ashworth (originally from the *Arabidopsis* Stock Centre, Nottingham, UK, and the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus, respectively). Seeds were surface sterilized with a solution composed of 50% commercial bleach and 0.1% SDS for 10 min and then rinsed five times with sterile distilled water. The seeds were germinated in Petri dishes containing Gamborg's B5 medium (GIBCO) solidified with 0.75% bactoagar (Difco). The plates were incubated initially at 4°C for 2 days and the fro 7 days under a 16-hr-lights/8-hr-dark photoperiod at 25°C. Seedlings were individually transferred into

baby food jars containing solidified B5 medium and grown for 7 to 10 days for root culture. Alternatively, the seedlings were transferred into soil for bolt inoculation.

[00082] **Growth of *Agrobacterium tumefaciens*.** All *Agrobacterium* strains were grown in YEP medium (Lichtenstein and Draper, 1986) supplemented with the appropriate antibiotics (rifampicin, 10μg/mL; kanamycin, 100 μg/mL) at 30°C. Overnight bacterial cultures were washed with 0.9% NaCl and resuspended in 0.9% NaCl a 2 x 109 colony-forming units per mL for in vitro root inoculation or at 2 x 1011 colony-forming units per mL for bolt inoculation.

In Vitro Root Inoculation and Transformation Assays Roots grown on the agar surface were excised, cut into small segments (~0.5 cm) in a small amount of sterile water, and blotted onto sterile filter paper to remove excess water. For some experiments, excised roots were preincubated on callus-inducing medium (CIM;4.32 g/L Murashige and Skoog [MS] minimal salts [GIBCO], 0.5 g/L Mes, pH 5.7, 1mL/L vitamin stock solution [0.5mg/mL nicotinic acid, 0.5 mg/mL pyridoxine, and 0.5 mg/mL thyamine-HCl], 100 mg/L myoinositol, 20 g/L glucose, 0.5 mg/L 2,4-dichlorophenoxyacetic acid, 0.3 mg/L kinetin, 5mg/L indoleacetic acid, and 0.75% bactoagar) for 1 day before cutting them into segments. Dried bundles of root segments were transferred to MS basal medium (4.32 g/L MS minimal salts, 0.5 g/L Mes, pH 5.7, 1 mL/L vitamin stock solution, 100 mg/L myoinositol, 10g/L sucrose and 0.75% bactoagar), and 2 or 3 drops of bacterial suspension were placed on them. After 10 min, most of the bacterial solution was removed, and the bacteria and root segments were cocultivated at 25°C for 2 days.

[00083] For transient transformation assays, the root bundles were infected with *Agrobacterium* strain GV3101 was used (Koncz and Schell, 1986) containing the binary vector pBISN1 (Narasimhulu *et al.*, 1996). After various periods of time, the roots were rinsed with water, blotted on filter paper, and stained with X-gluc staining solution (50 mM NaH2HPO4, 10 mM Na2 · EDTA, 300 mM mannitol, and 2 mM X-gluc, pH 7.0) for 1 day at 37°C. For quantitative measurements of β-glucuronidase (GUS) activity, the roots were ground in a microcentrifuge tube containing GUS extraction buffer (50 mM Na2HPO4, 5 mM DTT, 1 mM Na2 EDTA, 0.1% sarcosyl, and 0.1% Triton X-100, pH 7.0), and GUS specific activity was measured according to Jefferson *et al.* (1987).

[00084] To quantitate tumorigenesis, root bundles were infected with wild-type *Agrobacterium* strains. After 2 days, the root bundles were rubbed on the agar surface to remove excess bacteria and then washed with sterile water containing timentin (100μg/mL). Individual root segments (initial assay) or small root bundles (5 to 10 root segments; modified assay) were transferred onto MS basal medium lacking hormones but containing timentin (100μg/mL) and incubated for 4 weeks.

[00085] For transformation of root segments to kanamycin resistance, root bundles were inoculated with *Agrobacterium* strain GV3101 containing pBISN1. After 2 days, small root bundles (or individual root segments) were transferred onto CIM containing timentin (100μg/mL) and kanamycin (50μg/mL). Kanamycin-resistant calli were scored after 4 weeks of incubation at 25°C.

[00086] To determine stable GUS expression, roots were inoculated as given above and the root segments were transferred after 2 days to CIM containing timentin (100μg/mL) without any selection. After 4 weeks, GUS activity was assayed either by staining with X-gluc or by measuring GUS specific activity by using a 4-methylumbelliferyl β-D galactoside (MUG) fluorometric assay, as described above.

[00087] To determine the kinetics of GUS expression, root bundles were infected, the root segments were transferred after 2 days to CIM containing timentin (100μg/mL), and calli were grown on CIM without selection. Root bundles were assayed at various times, using a MUG fluorometric assay as described above, to measure GUS specific activity.

[00088] **Construction of simple binary vector systems.** The based constructs are monocot-optimized (e.g. utilize monocot promoters and enhancer introns) and contain proven selectable and screenable markers (e.g. *bar, gusA, gfp*). The *gusA* gene in all constructs contains an intron to prevent expression in *Agrobacterium*. A synthetic red-shifted, maize codon-optimized close *sgfp* (S65T) is (Chiu et al., 1996). The CaMV 35S promoter (double promoter region) is used to drive the screenable markers. The *bar* gene as a selectable marker (DeBlock et al., 1987). A maize ubiquitin promoter-intron (Ubi-1) is used to drive the *bar* gene expression (Christensen and Quail, 1996). the vectors are designed to reduce/eliminate the occurrence of repeated sequences within the constructs. If another promoter is needed, the "super-promoter" (Ni et al., 1995) is suitable. This promoter works well in maize does not have homology to the CaMV 35S or maize ubiquitin promoters, and

is freely available for licensing from the Biotechnology Research and Development Corporation. T-DNA border sequences and multiple cloning sites are included in the base constructs

[00089] **Construction of the binary vectors pKM4 and pKM5.** The plasmid pE1509 containing the 1.7 kbp junction fragment cloned into pBluescript was digested with EcoRI to release the junction fragment. The 5' overhanging ends were filled in using the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates. The T-DNA binary vector (pE1011) pGTV-HPT (Becker *et al.*, 1992) was digested with the enzymes SacI and SmaI, releasing the promoterless gusA gene from pGTV-HPT. The 3' overhanging sequence of the larger fragment containing the origin of replication and the hygromycin resistance gene (hpt) were removed using the 3'-5' exonuclease activity of Klenow DNA polymerase, and the resulting 1.7 kbp blunt end fragment was ligated to the blunt ends of the binary vector. A binary vector plasmid containing the 1.7 kbp fragment in the correct orientation (pAnos downstream of the histone H2A gene) was selected and named pKM4 (strain E1547).

[00090] An approximately 9.0 kbp wild-type genomic SacI fragment containing the histone H2A gene (*RAT5*) from a lambda genomic clone was cloned into the SacI site of the plasmid pBluescript. This 9.0 kbp SacI fragment was subsequently released from pBluescript by digestion with SacI and was cloned into the SacI site of the binary vector pGTV-HPT, resulting in the plasmid pKM5 (strain E1596). Both pKM4 and pKM5 were separately transferred by triparental mating (Ditta *et al.*, 1980) into the non-tumorigenic *Agrobacterium* strain GV3101, resulting in the strains A. tumefaciens At1012 and At1062, respectively.

[00091] **Germ-line transformation of *Arabidopsis*.** Germ-line transformations were performed as described in (Bent and Clough, 1998). Transgenic plants were selected on B5 medium containing hygromycin (20 μg/ml).

Agrobacterium tumefaciens Vector and Strain

[00092] *A. tumefaciens* strain EHA101 (Hood et al., 1986) containing the standard binary vector pTF102 (12.1 kb) was used in all experiments. The 5.9-kb T-DNA region of this construct is shown in Figure 1. The vector is a derivative of the pPZP binary vector (Hajdukiewicz *et al.*, 1994) that contains the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection. The

CaMV 35S promoter (P35S) was used to drive both the bar selectable marker gene and the gus reporter gene. A tobacco etch virus translational enhancer (Carrington and Freed, 1990) was included in the 5' end of the bar gene. The soybean (Glycine max L. Merrill) vegetative storage protein terminator (Mason et al., 1993) was cloned to the 3' end of the bar gene. The gus gene contained a portable intron in its codon region (Vancanneyt et al., 1990) to prevent GUS activity in *A. tumefaciens* cells. This vector system, pTF102 in EHA101, was maintained on yeast extract peptone (YEP) medium (An et al., 1988) containing 100 mg/L spectinomycin (for pTF102) and 50 mg/L kanamycin (for EHA101). Bacteria cultures for weekly experiments were initiated from stock plates that were stored for up to 1 month at 4°C before being refreshed from long-term, 80°C glycerol stocks. In all experiments, bacteria cell densities were adjusted to an optical density (OD_{550}) between 0.35 to 0.45 using a spectrophotometer immediately before embryo infection.

Plant Material

[00093] F_2 immature zygotic embryos (1.5-2.0 mm) of the maize (Zea mays) Hi II hybrid genotype (Armstrong et al., 1991) were aseptically dissected from greenhouse-grown ears harvested 10 to 13 d post pollination. Ears were stored up to 3 d at 4°C before dissection.

Media

[00094] Infection, cocultivation, resting, and selection media were after Zhao et al. (1999) except that cocultivation medium was modified to contain Cys. All these media contained N6 salts and vitamins (Chu et al., 1975), 1.5 mg/L 2,4-dichlorophenoxyacetic acid, and 0.7 g L1 L-Pro in addition to the following ingredients: infection medium contained 68.4 g/L Suc and 36 g/L Glc (pH 5.2) and was supplemented with 100 µM AS (Sigma, St. Louis) before use; cocultivation medium contained 30 g/L Suc, 0.85 mg/L silver nitrate, 100 µM AS, and 3 g/L gelrite (pH 5.8); resting medium contained 30 g/L Suc, 0.5 g/L MES, 0.85 mg/L silver nitrate, 250 mg/L cefotaxime, and 8 g/L purified agar (pH 5.8). Selection medium was identical to resting medium with the addition of 1.5 or 3 mg/L bialaphos (Shinyo Sanyo, Tokyo). Infection medium was filter sterilized, whereas all other media were autoclaved. AS stock solutions (100 mM) were prepared by dissolving AS in 100% (v/v) dimethyl sulfoxide (DMSO) to make a 200 mM stock which was then diluted (1:1 [v/v]) with sterile water and stored in small aliquots at 20°C. Cys was added to

cocultivation medium after autoclaving from freshly prepared, filter-sterilized stocks (100 mg/mL) and cocultivation medium was used within 2 to 5 d of preparation. Regeneration I medium contained Murashige and Skoog salts and vitamins (Murashige and Skoog, 1962), 60 g/L Suc, 100 mg/L myo-inositol, no hormones, and 3 g/L gelrite (pH 5.8) after Armstrong and Green (1985). Cefotaxime (250 mg/L) and bialaphos (3 mg/L) were added to this medium after autoclaving. Regeneration II medium differed from medium I in that it contained 30 g/L Suc and no bialaphos. All media was poured to 100- × 25-mL plates.

Infection and Cocultivation

[00095] *A. tumefaciens* cultures were grown for 3 d at 19°C on YEP medium amended with 100 mg/L spectinomycin and 50 mg/L kanamycin. One full loop (3 mm) of bacteria culture was scraped from the 3-d-old plate and suspended in 5 mL of liquid infection medium (Inf) supplemented with 100 μM AS (Inf + AS) in a 50-mL falcon tube. The tube was fixed horizontally to a bench-top shaker or a Vortex Genie platform head and shaken on low speed (approximately 75 rpm) for 4 to 5 h at room temperature. This pre-induction step was carried out for all experiments. For infection, immature zygotic embryos (1.5-2.0 mm) were dissected to bacteria-free Inf + AS medium (1.8 mL) in 2-mL eppendorf tubes (20-100 embryos per tube) and washed twice with this medium. The final wash was removed and 1 to 1.5 mL of A. tumefaciens suspension was added to the embryos. Embryo infection was accomplished by gently inverting the tube 20 times before resting it upright for 5 min with embryos submerged. Embryos were not vortexed at any time during this procedure. After infection, embryos were transferred to the surface of cocultivation medium and excess *A. tumefaciens* suspension was pipetted off the medium surface. Cocultivation medium contained 400 mg/L Cys unless stated otherwise. In experiments in which cocultivation medium treatments were compared, embryos were washed and infected in the same tube before being distributed between media treatments. Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up). Plates were wrapped with vent tape (Vallen Safety Supply, Irving, TX) and incubated in the dark at 20°C or 23°C for 3 d, after which embryos were transferred to 28°C on resting medium.

[00096] Embryo response (%) was measured as the number of cocultivated immature zygotic embryos that had initiated embryogenic Type II callus formation at their

scutellum base after 4 to 7 d on resting medium, compared with the total number plated. All embryos, responding or not, were transferred to selection medium.

Selection and Regeneration

[00097] After 4 to 7 d on resting medium (28°C, dark), embryos were transferred to selection medium (30 per plate) containing 1.5 mg/L bialaphos. Selection was increased to 3 mg L1 bialaphos 2 weeks later. Putatively transformed events were identified as early as 5 weeks after infection. Regeneration of R0 transgenic plants from Type II embryogenic callus was accomplished by a 2- to 3-week maturation step on Regeneration Medium I followed by germination in the light on Regeneration Medium II as described by Frame et al. (2000). Stable transformation efficiency (%) was calculated as the number of bialaphos-resistant callus events recovered per 100 embryos infected.

Acclimatization and Greenhouse Care of Transgenic Plants

[00098] Transplant and acclimatization of regenerated R0 plants was accomplished as described previously (Frame et al., 2000). Transgenic plants were grown to maturity in the greenhouse.

Statistical Analysis

[00099] Data from eight independent experiments were used to compare stable transformation efficiency from pairs of plates treated alike aside from Cys exposure during cocultivation. A sign test was used to determine whether the benefit in transgenic event recovery rate observed for the 400 mg/L Cys treatment was significantly higher than that for the 0 mg/L Cys treatment.

[000100] A Chi square test was used to determine whether the segregation ratios we observed for gus and bar gene expressing progeny plants fit the expected 1:1 ratio.

Histochemical Analysis of Transient and Stable gus Expression

[000101] Histochemical GUS assays (Jefferson, 1987) were used to assess transient expression of the gus gene in immature zygotic embryos 1 or 2 d after the 3-d cocultivation (4 or 5 d after infection). Level of transient gus expression was assessed on a per embryo basis by estimating the number of blue foci visible on the scutellum side of each embryo. Embryos displaying blue foci only on the embryo-axis side of the explant were scored as non-expressors. The embryo was then categorized as follows: nonexpresser (no blue foci), low expresser (one-25), moderate expresser (26-

100), or high expresser (>101). The number of embryos in each of these four groups was compared with the total number of embryos assessed to determine percent of total embryos in each of the expression categories. Histochemical GUS assays were also used to assess stable expression of the gus gene in bialaphos-resistant callus samples and in leaf tissue of transgenic plants in the R1 and R2 generations. Leaf segments (0.5 cm) were submerged in the substrate, vacuum infiltrated (20 inch Hg) for 10 min, and incubated at 37°C overnight. Blue staining cells were visualized by soaking leaf tissue in 75% followed by 95% (v/v) ethanol to remove chlorophyll and leaf pieces scored as positive or negative for GUS expression.

Southern-Blot Analysis

[000102] Leaf genomic DNA was prepared from 2 to 3 g of fresh leaf tissue from putative transgenic maize plants using the cetyltrimethylammonium bromide (CTAB) method, as described by Murray and Thompson (1980). Ten micrograms of genomic DNA per sample was digested with the HindIII restriction enzyme at 37°C overnight and separated on a 0.8% (w/v) agarose gel. DNA gel-blot analyses (Sambrook et al., 1989) were conducted on DNA samples using the 32P-labeled bar or gus fragments as shown in Figure 1.

Progeny Segregation Analysis for bar Gene Expression

[000103] A glufosinate leaf-spray test (Brettschneider et al., 1997) was used to establish segregation ratios for expression of the bar gene in progeny. The herbicide Liberty (Aventis, Strasbourg, France) was dissolved in water (1.25 mL/L) along with 0.1% (v/v) Tween 20 for a final glufosinate concentration of 250 mg/L. Beginning 9 d after planting, seedlings were sprayed three times at 1- to 2-d intervals with a freshly prepared glufosinate solution and then scored for herbicide resistance (alive) or herbicide sensitivity (dead).

Retransformation Experiments

A. T_2 immature zygotic embryos

[000104] Final results for experiments on T2 embryos from T1 seed derived plants of one A10 and one A11 event were presented in the last quarterly. This approach to assessing relative transformability of A10 vs A11 events was generally unsuccessful due to poor seed set on gh T2 ears.

B. A10S1 and A11S1 Callus

[000105] All the putative events picked from this callus bombardment experiment have been gus assayed and no blue was seen in any events. Recall however, that this Nobel Foundation construct, which carries the gus gene based on the map, did not produce any transients either (while the pBGF positive control bb alongside did).

[000106] These putative events were maintained on 50 or 75 mg/L hygromycin for weeks without dieing but without being convincingly real (clones being picked for section C below are as clear as a bell). BF took a subset of the best looking ones to regeneration to see if they will harden off. At present, it is likely that we recovered few to no clones from this massive amount of work. While no conclusions can be drawn about the benefit to biolistic transformation of the expression of the H2A protein in these lines, we can conclude that hygromycin selection for rapidly growing callus needs to be applied early and strongly to minimize excessive growth during subsequent selection.

C. A10 and A11 T1 embryos

[000107] Putative clones from T1 embryos of A10 (experimental) and A11 (control) T0 plants retransformed with pTOK233 have been picked, gus assayed and some named this quarter. The number of ears per A10 or A11 event, the numbers of embryos per ear infected and the number taken to hygromycin selection after resting the embryos on 2 mg/L bialaphos to kill-off the non-segregating embryos are summarized in Table 1. We have named 33 A10 putatives and 23 A11 putatives of which 15/17 and 10/22 of those assayed to date are gus positive, respectively. Using the number of embryos responding after resting on medium containing 2 mg/L bialaphos (to kill non A10 or A11 segregating T1 embryos), the overall efficiency for A10S1 retransformation using A18S6 was 33 events/401 infected, non-dead embryos (8.2%) and for A11S1 was 23 events/ 753 embryos (3%) (Table 2). This suggests that, over 16 A10S1 ears and 17 A11S1 ears, the average efficiency of transformation was higher for A10 than A11 in this screen.

[000108] Table 2. Summary of retransformation experiment

Cross ID	Northern expression levels in original event	total # of ear	total infected by event	total resp by vent	total putatives by event	Overall efficiency
H2A expressing lines:						
A10S1-44 X B73	LOW	4	202	104	0	0.0
A10S1-46 X B73	HIGH	5	269	128	8	6.3
A10S1-47 X B73	HIGH	1	30	12	2	16.7
A10S1-48 X B73	LOW	2	107	26	5	19.2
A10S1-49 X B73	MOD-HIGH	2	141	75	11	14.7
A10S1-51 X B73	MOD	2	101	56	7	12.5
Total		**16**	**850**	**401**	**33**	**8.2**
Vector control lines:						
A11S1-27 X B73	NONE	2	261	136	2	1.5
A11S1-30 X B73	NONE	3	275	184	7	3.8
A11S1-31 X B73	NONE	5	395	211	7	3.3
A11S1-33 X B73	NONE	3	270	141	7	5.0
A11S1-34 X B73	NONE	4	224	81	0	0.0
Total		**17**	**1425**	**753**	**23**	**3.1**

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